ANTICANCER EFFECTS OF METHYLATED AND NONMETHYLATED SOY ISOFLAVONES IN PRECANCEROUS PROSATE CELLS

A Senior Honors Thesis

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Abstract

Prostate cancer is the most frequently diagnosed form of cancer and the second leading cause of cancer related deaths in the United States. Increased consumption of soy is thought to reduce the risk for this disease. More specifically, the isoflavones found in soy are responsible, in part, for these anticancer effects. Isoflavones are organic compounds found in soy and other legumes and it is thought that methylated isoflavones (glycitein, biochanin A, formononetin) may have greater anticancer activity than those without methyl groups (genistein, daidzein, equol). However, the majority of studies, to date, have focused primarily on the nonmethylated isoflavones, genistein and daidzein. Epidemiological evidence also suggests that the anticancer effects of soy may be greatest during the precancerous stages of prostate cancer. Few studies, however, have examined the impact of soy isoflavones during this precancerous stage. This study examined the antiproliferative effects of methylated and nonmethylated soy isoflavones using a precancerous prostate cell line (WPE1-NB14). The precancerous prostate cells were treated with the six different soy isoflavones, three methylated and three nonmethylated, in different concentrations (0-50μM). Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. This assay uses MTT to color the living cells in order to determine the cell viability. The results of this study suggest that methylated isoflavones reduced precancerous cell viability to a greater extent than nonmethylated isoflavones and indicate that the methyl group does contribute to the anticancer effects of soy isoflavones in precancerous prostate cells. While most studies focus on nonmethylated isoflavones because they are the most abundant, they are not necessarily the most bioactive. This study demonstrates the positive impact methylated isoflavones can have on prostate cancer prevention.

Introduction

Prostate cancer is the most frequently diagnosed and the second leading cause of cancer related deaths in the United States (1). The incidence of prostate cancer in Asian populations, however, is tenfold lower than that of western cultures. These observed differences in prostate cancer incidence are thought to be due, in part, to increased consumption of soy in Asian countries (2). Isoflavones are the specific compounds found in soy that are thought to contribute to the reduction of prostate cancer in men. The predominant isoflavones found in soy are genistein, daidzein, and glycitein, comprising 50, 40, and 10% of the total isoflavone profile, respectively. Although these compounds are structurally similar, studies suggest that each individual isoflavone inhibits cancer processes such as proliferation via different mechanisms. For example, genistein has been shown to inhibit proliferation at high concentrations yet induce proliferation at low concentrations in prostate cells (3). Studies on glycitein, however, show that this isoflavone significantly reduces the proliferation of prostate cells at lower concentrations than other soy isoflavones (4). Glycitein is unique in that it is the only isoflavone found in soy with a methoxy group.

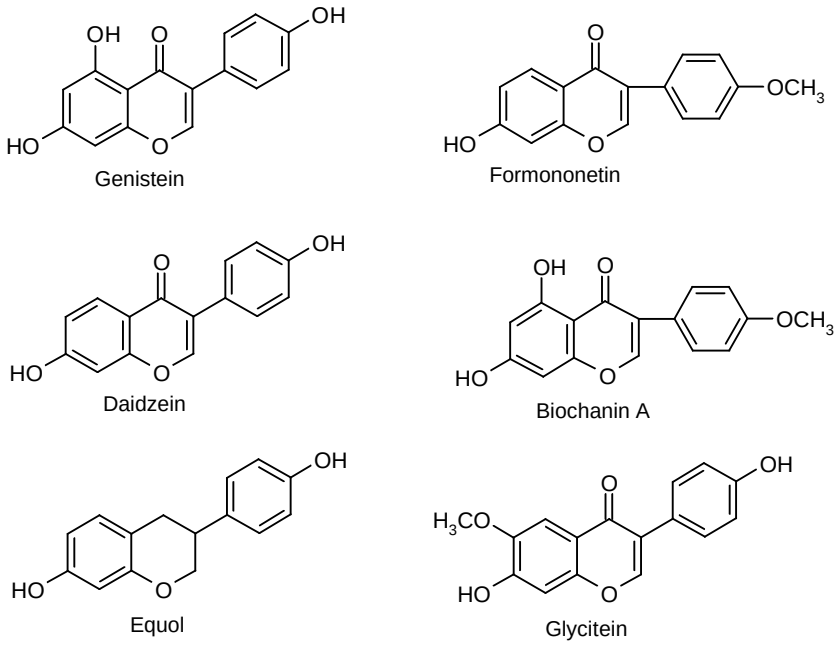

Figure 1. Isoflavone Structures

Interestingly, methylated flavones have been shown to be more bioavailable and biologically stable than nonmethylated flavones (5-6). However to date, the anticancer effects of methylated isoflavones such as glycitein found in soy, and biochanin A and formononetin found in red clover, have not been well characterized. This study examined the antiproliferative effects of methylated and nonmethylated isoflavones using a precancerous prostate epithelial cell line (WPE1-NB14). **The central hypothesis of this research is that methylated isoflavones reduce the cell viability of the WPE1-NB14 cell line to a greater extent than the nonmethylated isoflavones.**

Literature Review

Prostate cancer is the most diagnosed and the second leading cause of cancer related death among American males (1). The single most predictive risk factor for prostate cancer is age. Therefore, as life expectancy increases, the total number of predicted cases also increases (7). Prostate cancer development can take years or even decades to manifest and its development occurs in stages of premalignant lesions called prostatic intraepithelial neoplasia (PIN). Approximately one third of PIN cases develop into prostate cancer and its presence in the prostate greatly increases the risk of acquiring this disease (7). PIN is subsequently divided into two grades, low grade PIN (LGPIN) and high grade PIN (HGPIN). LGPIN is characterized by irregular spacing

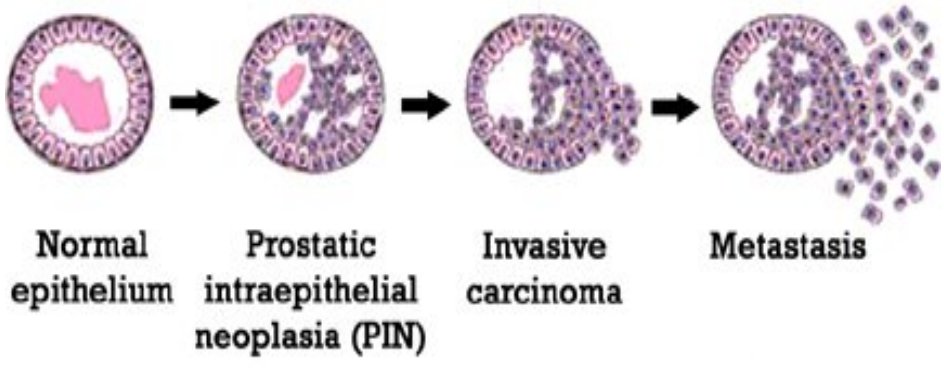

Figure 2. Progression of Prostate Cancer
(http://rwjms2.umdnj.edu/department/division/faculty/index.htm)

of epithelial cells, cell growth and stratification (7) and it has been observed in men in their 20s (8). HGPIN is characterized by even more epithelial cell crowding and stratification (7) and has been observed in men as early as 40 years old (8-9). Due to the prolonged latency of PIN, strategies that target this precancerous lesion in the prostate may reverse or delay the onset of prostate cancer.

American and European males have a tenfold increase in the risk of prostate cancer development as compared with East Asian countries (10-11). The observed difference in the prevalence of prostate cancer among Asian men and American men may be due, in part, to differences in soy consumption. Interestingly, Japanese men that immigrate to the US appear to have a higher risk for prostate cancer than those who remained in Japan (12). This observation suggests that environmental factors, such as diet, play a pivotal role in the risk for prostate cancer. Several epidemiological studies have been conducted to examine the effect of soy consumption on the prevalence of prostate cancer. Kurahashi et al. (2) showed an inverse relationship between the amount of soy consumed in the diet and the risk of developing prostate cancer in Japanese men. It has been widely thought that isoflavones found in soy contribute, in part, to the reduced risk of prostate cancer. In fact, men with the highest intake of isoflavones had a reduced risk for prostate cancer compared to the men with lower isoflavone intakes (2). Japanese men consuming a traditional diet tend to have ten to one hundredfold greater concentrations of plasma isoflavones than Western men (10-11) that do not consume soy.

Studies suggest that increased consumption of soy reduces the risk of HGPIN and prostate cancer rather than the initial LGPIN lesions (2). The incidence of LGPIN appears to be equivalent in both American and Japanese men; however, a greater incidence of HGPIN and prostate cancer is observed in American men (13-14). These observations suggest that

consumption of soy is most beneficial during the precancerous stages of the prostate carcinogenic process. In fact, several epidemiological studies suggest that a diet rich in soy and soy isoflavones may prevent prostate cancer during noncancerous and precancerous stages of the carcinogenic process and that during advanced disease, exposure to soy isoflavones may no longer be beneficial (14-17).

The anticancer effects of genistein and daidzein have been extensively studied in prostate cancer cell models (18-27). Although structurally similar, genistein and daidzein differ in the intensity and mechanisms of inhibiting tumor and cellular growth and proliferation. Several studies show that low concentrations of genistein increase cell proliferation while higher concentrations greatly inhibit proliferation (3, 18). Furthermore, genistein has been shown to induce cell cycle arrest in the G2 phase as well as increase apoptosis (28). However, daidzein and a metabolite of daidzein, equol, have been shown to reduce cell proliferation in prostate cancer cells (29) and induce cell cycle arrest in G0/G1 phase (30) rather than the G2 phase as observed with genistein. In vivo studies show that genistein reduces the formation of poorly differentiated tumors in the prostate of a transgenic mouse model (TRAMP). This reduction in tumors was greatest in the mice that were exposed to genistein throughout life (27).

The anticancer effects of the soy isoflavone glycitein have not been well studied. However, studies report that this minor soy isoflavone may be more bioactive than genistein and daidzein (4, 31). Glycitein has been shown to significantly reduce the proliferation of noncancerous prostate cell lines at concentrations tenfold less than genistein and daidzein. Interestingly, glycitein is the only isoflavone with a methoxy group. Studies suggest that flavones with methoxy groups appear to have more beneficial qualities than their nonmethylated counterparts including the enhanced ability to resist metabolism, a greater biological stability,

and improved intestinal transport (5-6). However, the bioactivity and anticancer activity of methylated isoflavones have not been extensively explored. Although glycitein is the only soy isoflavone with a methoxy group, other methylated isoflavones are found in plant material such as red clover. Methylated isoflavones (glycitein, biochanin A, formononetin) may have greater anti-cancer activity than those without methyl groups (genistein, daidzein, equol) but this difference in isoflavone structure has not yet been studied.

Most in vitro studies examining the anticancer effects of soy isoflavones have utilized prostate cancer cell lines. However, the epidemiological evidence suggests that the beneficial effects of soy are greatest during precancerous stages of the prostate carcinogenic process. In fact, several dietary bioactive compounds have been shown to be most effective of specific stages during cancer (4, 32-34). Although the literature provides invaluable information regarding the anticancer effects of soy isoflavones in cancer models, the anticancer effects of isoflavones in precancerous models have not been well characterized. Therefore, this study examined the antiproliferative effects of methylated and nonmethylated isoflavones using a precancerous prostate epithelial cell model. The WPE1-NB14 cell line mimics the precancerous stages of the prostate carcinogenic process. This cell line was developed from the noncancerous RWPE-1 cell line. RWPE-1 cells were developed from epithelial prostate cells and exposed to human papilloma virus-18 which enabled them to mimic prostate cells and their responses to hormones (35). Briefly, RWPE-1 cells were exposed to N-methyl-N-nitrosourea (MNU). The carcinogen, MNU, enables these cells to imitate cells in the early stages of cancer (35).

Methods

Cell Culture: The human prostate precancerous epithelial cell line (WPE1-NB14) was obtained from the American Type Culture Collection (Rockville, MD). WPE1-NB14 cells were maintained in keratinocyte serum-free medium (GIBCO Laboratories, Grand Island, NY) supplemented with 50µg/ml bovine pituitary extract, 5% L-glutamine, 5ng/ml epidermal growth factor and penicillin/streptomycin (1.0%). This cell line was maintained in a humidified incubator (5% CO_2, 95% O_2) at 37ºC.

Proliferation: Cells were plated in 48-well plates at an initial density of 8.0×10^3 cells per well with supplements. Cells were treated with or without glycitein, biochanin A, formononetin, genistein, daidzein and equol (0-50µM) and incubated for an additional 72 hours. After incubation, cell proliferation will be determined by the 3-[4,5-dimethylthiazol-2-yl]-2-,5-diphenyltetrazolium bromide (MTT) assay. MTT was dissolved in PBS at 3mg/ml. Briefly, 15 µl of MTT solution was added to each well, followed by a three hour incubation. After incubation, MTT-containing medium was removed and 150µl of 0.04M HCl in isopropanol was added to each well to dissolve formazan crystals. The concentration of formazen was quantified spectrophotometrically at 595nm.

Statistical Analysis: Statistical significance between groups was determined by one-way analysis of variance with Tukey's post-hoc comparisons (SigmaStat software; Chicago, IL). Data are presented as means ± relative standard error with alpha $p<0.05$ considered significant.

Results

The methylated isoflavones reduced cell viability to a greater degree than the nonmethylated isoflavones. Figure 3A shows that biochanin A significantly reduced cell viability by 21% and 47% at 25µM and 50µM, respectively. Figure 3B shows that formononetin significantly reduced cell viability by 36%, 42% and 47% at 12.5µM, 25µM, and 50µM, respectively. Figure 3C shows that the methylated isoflavone, glycitein, had a significant reduction in cell viability by 26%, 24% and 48% at 12.5µM, 25µM, and 50µM, respectively. All of the methylated isoflavones had a significant reduction of cell viability in the 25µM and 50µM concentrations, with the greatest reduction in the 50µM. Of the nonmethylated isoflavones, genistein had a significant reduction in cell viability by 19% at 50µM (figure 3D). Similarly, figures 3E and 3F show that daidzein and equol significantly reduced cell viability at 50µM by 16% and 17%, respectively.

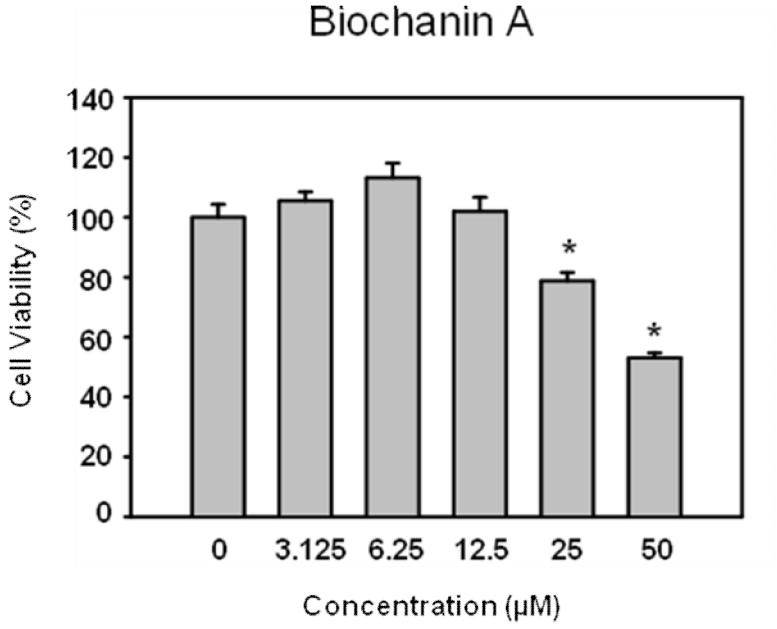

Figure 3A. Effects of biochanin A on WPE1-NB14 cell viability. Cells were treated (72 hours) with biochanin A (0-50µM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

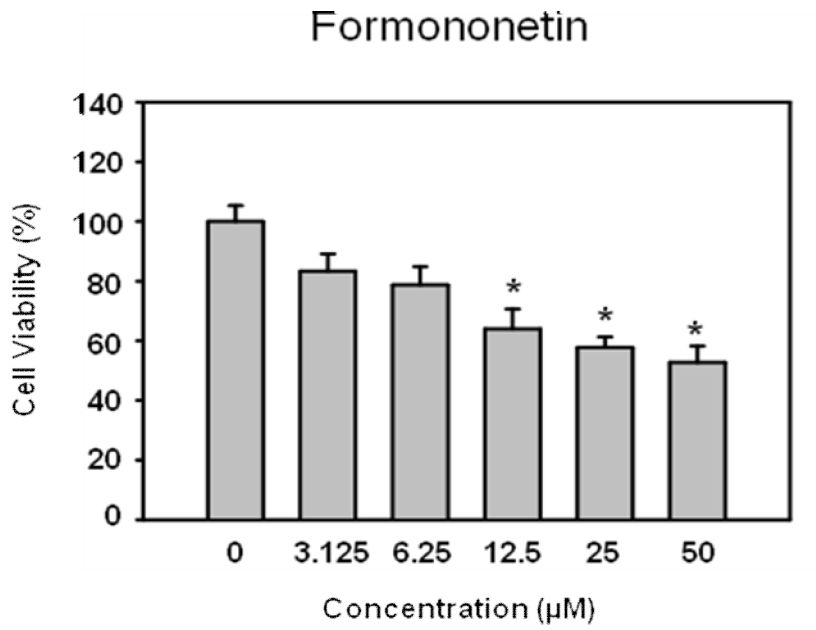

Figure 3B. Effects of formononetin on WPE1-NB14 cell viability. Cells were treated (72 hours) with formononetin (0-50μM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

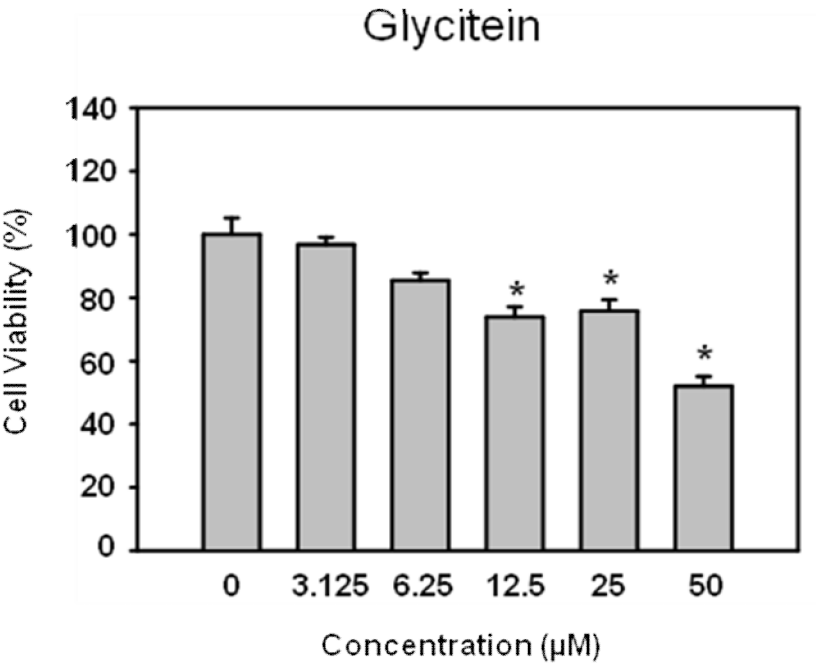

Figure 3C. Effects of glycitein on WPE1-NB14 cell viability. Cells were treated (72 hours) with glycitein (0-50μM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

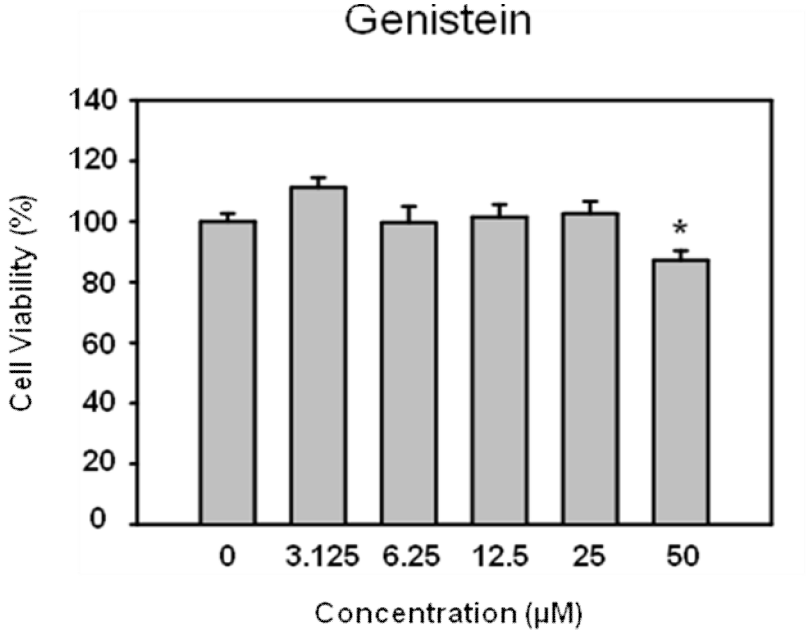

Figure 3D. Effects of genistein on WPE1-NB14 cell viability. Cells were treated (72 hours) with genistein (0-50µM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

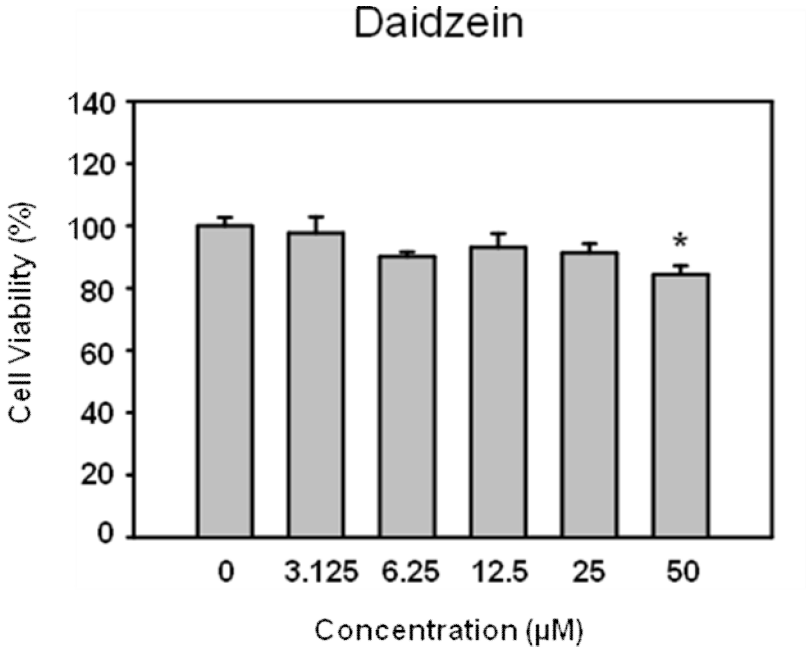

Figure 3E. Effects of daidzein on WPE1-NB14 cell viability. Cells were treated (72 hours) with daidzein (0-50µM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

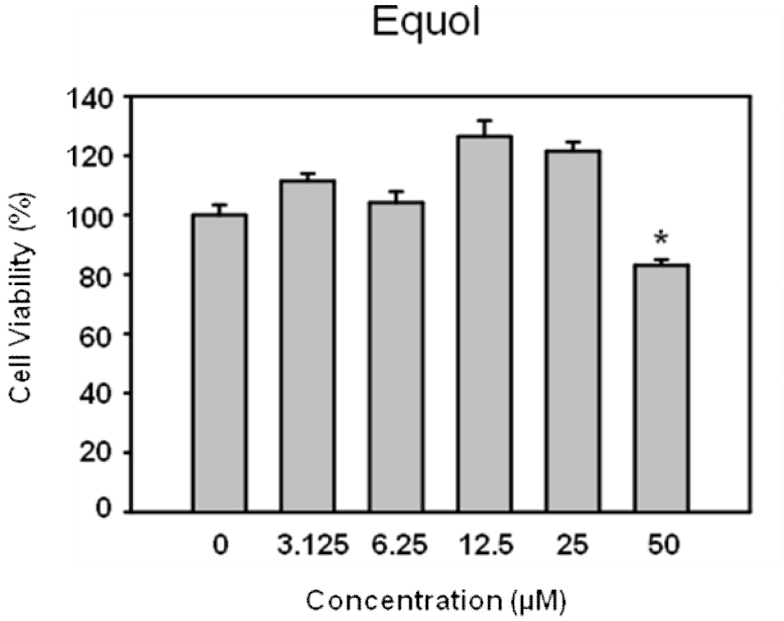

Figure 3F. Effects of equol on WPE1-NB14 cell viability. Cells were treated (72 hours) with equol (0-50µM) and cell viability was determined by MTT assay. * indicates a significant ($p<0.05$) reduction in viability compared to control.

Discussion and Conclusions

The results of this study show that methylated isoflavones reduced precancerous cell viability to a greater extent than nonmethylated isoflavones. The structural differences of the isoflavones may be responsible, in part, for these results. Further research is needed to determine the mechanisms of these different isoflavones.

Isoflavones are found mainly in legumes such as soy, lentils, beans and chickpeas (36). Soy, specifically, contains considerable amounts of genistein, daidzein and glycitein. Biochanin A and Formononeitin are the isoflavones found in significant amounts in red clover (37). Other sources of isoflavones are alfalfa roots (formononetin) and chickpeas (biochanin A) (38). It has also been found that animal products, not only plant based products, contain isoflavones (39). However, these foods such as milk, eggs, and beef contain significantly less isoflavones than

plant based foods (39). The possibility of introducing isoflavones into non-legumes has been suggested and with the decreasing costs of DNA sequencing, it is more possible now than ever before (38).

Another direction for further research might be in observing how isoflavones impact precancerous prostate cell growth in vivo. When methylated isoflavones, biochanin A and formononetin are ingested, they are absorbed into the gastrointestinal tract and travel through the portal vein to the liver. In the liver, these isoflavones are demethylated and become their nonmethylated derivatives, genistein and daidzein. Only then do they enter the circulatory system and eventually reach the prostate (37). While these isoflavones are not in their most bioavailable form when they reach the prostate, it might be possible for the isoflavones to be remethylated enabling them to have a greater impact on cell viability. This might be achieved by the prostate's own mechanisms or potentially by ingestion of a methyl donor such as S-sdenosyl-L-methionine (SAMe). SAMe is a nutritional supplement that is involved with many transmethylation reactions in the liver (40) as well as in the creation of the essential phospholipid, phosphatidylcholine, and neurotransmitters including epinephrine and dopamine (41). This study demonstrates that there is a significant difference in the biological effects of methylated and nonmethylated isoflavones on WPE1-NB14 cells and will hopefully facilitate further research in this area.

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